Differentiation of persistent anatomical defensive structures is costly and determined by nutrient availability and genetic growth-defence constraints

Running title: **LIMITED NUTRIENT AVAILABILITY ENHANCES RESIN CANALS**

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ABSTRACT

Conifers exhibit a number of chemical and anatomical mechanisms to defend against pests and pathogens. Theory predicts an increased investment in plant defences under limited nutrient availability, but while this has been demonstrated for chemical defences, it has rarely been shown for anatomical defensive structures. In a long-lived woody plant we tested the hypothesis that limited nutrient availability may promote an improved differentiation of persistent anatomical defences. We also hypothesized that the costs of differentiation of those long-term anatomical structures may be determined by genetic constraints on early growth potential. Using *Pinus pinaster* juveniles, we performed a greenhouse study with 15 half-sib families subjected to experimental manipulation of P availability and herbivory-related induced responses. When plants were ca. 30 cm high, half of the plant material was treated with methyl jasmonate (MJ) to induce defences, and two weeks later plants were harvested and the abundance of resin canals in the cortex and xylem were assessed. Density of constitutive resin canals in the cortex and the total canal system were about 1.5-fold higher in plants under limited P availability than in fully-fertilized plants. Availability of P did not significantly influence inducibility of resin canal traits. We found negative genetic correlations between plant growth and the density of constitutive canals in the xylem and total canal system, but only under conditions of limited nutrition. These results demonstrate for the first time that differentiation of constitutive anatomical-based defences is affected by P limitation. Moreover, results also evidence the existence of genetic constraints between the plant growth and constitutive defensive investment, where lineages with the highest growth potential showed the lowest investment in constitutive resin canals.

Key words: *growth-defence trade-offs · resource allocation · traumatic resin ducts · growth-differentiation balance hypothesis · resource availability hypothesis*

INTRODUCTION

Conifers, the largest and longest-lived plants on the planet, have evolved potent and effective defensive mechanisms against a vast number of herbivores and pathogens (Schulman 1954). Conifer defences are largely based on the combination of high concentrations of a diverse array of carbon-based compounds (i.e. chemical defences) and physical structures (i.e. defences of an anatomical nature) which reduce the impact of their enemies (Franceschi et al. 2005, Mumm and Hilker 2006). These defences can also be classified as constitutive (always present in the plant) or inducible (produced in reaction to biotic challenges), the latter presumably having evolved in response to differences in the strength and variability of challenging factors (Karban 2011). Induced defences are expected to be more cost efficient than constitutive defences, as the former are only produced when required in response to environmental cues. Constitutive defences, on the other hand, are supposedly favoured where there is a high risk of herbivory.

It is well-known that production of constitutive chemical defences varies principally in response to diverse abiotic factors such as soil nutrient availability (Orians et al. 2003, Cipollini and Heil 2010, Heil 2010). It is also widely accepted that plants invest relatively more energy in constitutive defences when living in resource-poor conditions than in resource-rich conditions. Based on comparisons of defensive phenotypes across species, the Resource Availability Hypothesis (RAH) predicts that plants adapted to resource-poor and stressful habitats will be slow-growing and consequently invest significantly in constitutive defences, because their high tissue

84value means that losses due to herbivory are more costly (Coley et al. 1985, Endara and
85Coley 2011). Similarly, based on the physiological trade-off between growth and
86chemical defences, and on conflicts between biosynthetic pathways, the Growth-
87Differentiation Balance Hypothesis (GDBH) predicts that under resource impoverished
88conditions growth is limited more than photosynthesis, and the consequent excess of
89carbon is diverted to the production of defences (Herms and Mattson 1992). The
90environment is also though to also modulate the expression of induced defences
91(reviewed by Cipollini and Heil 2010), but empirical evidences for this is still limited
92and sometimes inconsistent, especially in long-lived plant species. For instance, induced
93investment in chemical defences was found to be greater under nutrient-limiting
94conditions in young pine trees (Sampedro et al. 2011), while increase in resin flow in
95response to mechanical damage was found to be constrained by drought stress in mature
96pine trees (Lombardero et al. 2000).

97 Trade-offs between the allocation of resources to constitutive defences versus
98other plant functions such as growth have been reported in greenhouse and field studies
99for several herbaceous (reviewed by Koricheva 2002) and woody plants (Zas et al.
1002005, Donaldson et al. 2006, Osier and Lindroth 2006, Sampedro et al. 2011). In
101contrast, and probably due to the difficulty of assessment, much less is known about the
102existence of genetic based trade-offs (i.e. negative genetic correlations) associated with
103the expression of induced defences (Cipollini et al. 2003, Cipollini and Heil 2010),
104especially in trees (but see Sampedro et al. 2011). Such negative correlations between
105growth potential and the expression of constitutive or induced defences reflect the costs
106of defences in terms of fitness, and are evidence of the heritable, genetic trade-offs
107between life functions, and are therefore of evolutionary relevance (Agrawal et al.
1082010, Sampedro 2014). Genetic trade-offs between growth and defence (either

constitutive or induced) might be also strongly modulated by environmental conditions. Several authors have observed that these trade-offs only emerge under limiting soil nutrient availability, and are weak or absent when conditions are more favourable for growth (e.g. Osier and Lindroth 2006, Donaldson and Lindroth 2007, Sampedro et al. 2011).

Increased production of chemical defences in secretory cells and tissues in response to biotic stress is commonly accompanied by the differentiation of physical barriers and anatomical structures of defence. In particular, pine trees produce numerous constitutive and inducible resin canals in their cortex and xylem tissues, forming a network of storage ducts surrounded by secretory parenchyma (Franceschi et al. 2005). This network supplies a copious resin flow in the wounding sites, which acts as the first barrier against herbivores and pathogens (Krokene et al. 2003, Franceschi et al. 2005, Mumm and Hilker 2006, Solla et al. 2006). Differentiation of such anatomical defensive structures in pine trees is persistent and irreversible, and it depletes large amounts of carbohydrate reserves (e.g. Bonello et al. 2006). The formation of canals involves slow and expensive processes such as cell division and differentiation, as opposed to chemical defences that usually involve rapid plastic changes in the metabolism of secretory cells (Bonello et al. 2006). Therefore, the effects of the environment on the allocation of resources for anatomical defensive changes, and the associated costs in terms of vegetative fitness, might be expected to be greater than those previously observed for chemical defensive changes. However, while the influence of nutrient availability on the defensive phenotype and on their costs has been well documented for chemical defences (Koricheva 2002, Donaldson et al. 2006, Osier and Lindroth 2006, Sampedro et al. 2011), it has been rarely reported for anatomical defensive traits.

The aim of this study was to investigate the effects of limited nutrient availability on the differentiation between constitutive and induced anatomical defensive structures (namely the resin canal system), using pine trees as a long-lived woody plant model. We investigated the genetic trade-offs between growth and resin canal formation, that are symptomatic of the vegetative costs associated with defence. We also examined genetic variation in constitutive and induced anatomical defences, and in the response of these defences to P availability. We performed a greenhouse experiment with *Pinus pinaster* Ait. (Pinaceae) half-sib families where P availability and herbivore-induced responses were experimentally manipulated. Herbivore-induced responses were mimicked by applying methyl jasmonate (MJ), a plant hormone involved in the signalling of defensive responses against chewing herbivores and necrotrophic pathogens (Thaler et al. 2012). We focused on soil P availability because P is the most limiting nutrient for growth of young *P. pinaster* trees in our study area (Martíns et al. 2009) and because P was previously reported to drive herbivore damage and resistance in young *P. pinaster* plantations (Zas et al. 2006, Moreira et al. 2008). We hypothesized (i) that pine juveniles growing under P-limited conditions would show higher constitutive and induced differentiation of resin canals compared to pines growing under complete fertilization, (ii) that there would be genetic variation between families in constitutive and induced resin canal traits and in their response to P availability (iii) that the differentiation of resin canals would trade-off with growth, and (iv) that the vegetative costs associated with producing anatomical defences, expressed as negative family correlations between those traits, could be context-dependent, and they would be more relevant under limited P availability than under complete fertilization.

MATERIAL AND METHODS

Natural history

Pinus pinaster is a conifer species of great economic and ecological relevance in southern Europe and the Mediterranean basin. The distribution range of this species extends from Portugal and northern Spain to south-western France, Italy, and Morocco (Richardson 1998). It generally occurs at low to moderate elevations, mostly from sea level to 600 m (Richardson 1998).

As with other conifer species, *P. pinaster* has a well-defined defensive arsenal against a broad range of ecologically important herbivores and pathogens (Moreira et al. 2008, Sampedro et al. 2011). This defensive arsenal is mostly based on the constitutive and induced production of resin and phenolic compounds (Sampedro et al. 2011). Both types of secondary compounds are present in large concentrations in all tissues along the plant, imposing ecologically-relevant physiological costs (Mumm and Hilker 2006, Sampedro et al. 2011, Moreira et al. 2014, Villari et al 2014).

Conifer resin is a viscous liquid composed of terpenoids, of which about 50% are diterpenes (C_{20}) and diterpene acids (called resin acids) that make resin thick and sticky (Phillips and Croteau 1999, Franceschi et al. 2005, Bohlmann 2008). The remaining 50% is a variable mixture of volatile terpenes (monoterpenes –C_{10}– and sesquiterpenes –C_{15}–) that serve as a solvent for the transport of non-volatile diterpenes (Phillips and Croteau 1999, Franceschi et al. 2005, Bohlmann 2008). Conifer resin is constitutively synthesized and accumulated in specialized secretory structures, such as resin canals (ducts), resin blisters, and resin cavities (Phillips and Croteau 1999, Trapp and Croteau 2001). Constitutive axial resin canals appear both in the xylem and the cortex following a scattered distribution. Herbivore wounding and fungal infection in pine trees elicit local and systemic damage signalling in which jasmonic acid

184derivatives are known to be crucial (Martin et al. 2002, Krokene et al. 2008, Miller et
185al. 2005). In response to damage, pine trees can develop traumatic resin canals in the
186secondary xylem which are usually found in tangential series of one or two rows within
187an annual ring of xylem (Krokene et al. 2003, Franceschi et al. 2005). The defensive
188function of the resin canal system is well documented, and more dense resin canals
189have been associated with improved field resistance to insect pests and pathogens
190(Kane and Kolb 2010, Moreira et al. 2012a, Ferrenberg et al. 2014).
191
192**Experimental design**
193A controlled greenhouse experiment with pine genetic entries, soil phosphorus (P)
194fertilization, and induction of defences as the main factors was carried out. The
195experiment followed a randomized split-split design replicated in four blocks, with soil
196P fertilization (two levels; complete and P-limited) as the whole factor, treatment of
197defence induction (two levels; control [i.e. constitutive] and MJ-induced plants) as the
198split factor, and fifteen genetic entries (open-pollinated half-sib families of known
199mother trees) as the split-split factor. In total, there were 240 plants corresponding to 4
200blocks × 2 soil phosphorus treatments × 2 induction treatments × 15 genetic entries.
201
202**Plant material, culture and greenhouse conditions**
203*Pinus pinaster* families were randomly selected from a broader collection of mother
204trees belonging to the Atlantic coast population of Galicia (NW Spain). A description of
205climate, soil characteristics, genetic variation in resistance and other characteristics of
206the study area and pine population can be consulted in Sampedro et al. (2011). The 15
207open-pollinated pine families studied here were a random subset of the 34 families
208included in Sampedro et al. (2011), a companion paper that focused on chemical

defences in stems and needles of the same trees. In February 2006, pre-weighted pine seeds were individually sown in 2-L pots filled with a mixture of perlite and peat (1:1 v:v) and covered with a 1-2 cm layer of sterilized sand. To avoid pathogens, seeds were preventively treated before sowing with a fungicide (Fernide®, Syngenta Agro, Spain). All plants were grown in a research grade glasshouse (Forestry Research Centre of Lourizán, Xunta de Galicia, Pontevedra, Spain), with controlled temperature (10ºC minimum temperature at night; 25ºC maximum temperature during daytime) and light (minimum 12 h per day). Fungicide (Fernide® Syngenta Agro S.A.) was applied to the substrate every two months.

Treatments of fertilization and induction of defensive responses

One month after sowing, the fertilization treatments (complete and P-limited fertilizer) were applied every two days by subirrigation. The complete fertilizer was a balanced solution containing 100:20:70:7:9 mg L^{-1} of N:P:K:Ca:Mg respectively, and the necessary amounts of micronutrients and trace elements. This solution was a modification of that used by local nurseries for optimum seedling growth of this pine species. The P-limited fertilizer solution contained the recommended levels of N, K, Ca and Mg, as described above, but the availability of P was reduced 10-fold to 2 mg L^{-1}. Fertilizer solutions were prepared every two weeks, and pH was adjusted to 6.5 in both treatments.

In August 2 2006, when average height of P-limited and complete fertilized plants were 21.9 ± 0.7 cm and 44.3 ± 1.3 cm respectively, 120 plants (i.e. half of the total number) were individually treated with a solution of 22 mM methyl jasmonate (Sigma-Aldrich, St. Louis, MO, USA) suspended in deionized water with ethanol 2.5% (v:v). The remaining 120 plants were treated with the carrier solution (2.5% ethanol)

and acted as control. Treatments were sprayed evenly over the foliage with a handheld sprayer, each plant receiving 2.6 ± 0.2 or 3.7 ± 0.3 mL of solution (P-deficient and complete fertilization plants, respectively). To avoid cross-contamination, treatments were applied in separate greenhouse chambers and plants remained in those separate spaces for 24 h to dry.

Sampling, measurements and histological analyses

Two weeks after application of the induction treatment, plant height was measured and all pine juveniles were harvested, transported to the lab in ice coolers, and immediately sampled for histological analyses and total biomass determination. A 5-cm long subsample of the apical portion of the stem from each plant was immediately placed in a glass vial and fixed in formalin – acetic acid – ethyl alcohol for approximately 48 h according Moreira et al. (2008) and then transferred to 70 % ethyl alcohol for storage until sectioning and staining. Cross-sections (90 μm thick) were made using a sliding microtome. Sections were stained with 0.1% aqueous Safranin and Fast-Green according standard procedures (Ruzin 1999, Moreira et al. 2008). Photographs were taken with a Nikon Digital Sight DS-U1, mounted on a Nikon SMZ-U binocular microscope at x20 magnification. Image analysis was performed on a quarter of cross section as in Moreira et al. (2012a) using the Phloemalizer v.2.12 image analysis software developed at the Pacific Forestry Centre (British Columbia Forest Service, Victoria, BC, Canada).

The resin canal system of cortex and xylem, as well the total canal system (i.e. sum of cortex and xylem), was characterised in terms of (i) resin canal density (the number of axial resin canals per unit area), and (ii) relative conductive area (%),

obtained by dividing the total transectional area occupied by the resin canals by the total area of the tissue assessed, and then multiplying by 100 (Moreira et al. 2008).

Statistical analyses

Preliminary analyses of the effects of induction treatment (control vs. MJ application) on growth and resin canal traits were carried out with a proper mixed model to solve split-split designs (Littell et al. 2006) using the PROC MIXED procedure of SAS System (version 9.2, SAS Institute, Cary, NC). These models included the fixed effects of Phosphorus availability (P), defence induction treatment (T), and their interaction, and the random effects of the Family (F), and its interaction with the main effects, as well as the random effects of the blocks (B), and the B×P and B×P×T interactions.

Analyses of constitutive resin canal traits (control untreated plants) and of their inducibility after MJ application (see below) were also carried out independently with a mixed model using the PROC MIXED procedure of SAS (Littell et al. 2006). The main effect of soil phosphorus availability (P) was considered as a fixed factor. The block (B) and B × P interaction were considered random factors in order to analyse the main factor P with the appropriate error term. Family (F) and P × F interaction were also considered as random factors, and associated variance components were estimated by restricted maximum likelihood. The statistical significance of the variance components for each random factor was assessed using likelihood ratio tests, where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one tailed χ^2, with one degree of freedom (Littell et al. 2006). Normality was achieved by log-transforming the raw data.

Because resin canal traits in induced plants are a result of the sum of pre-existing constitutive levels plus the induced response, variability in the induced mode

would not properly represent the variation in inducibility, i.e. in the ability to increase defences after induction. Thus, in order to test for the effect of P availability and genetic variation on inducibility *per se,* we analysed our data using a bootstrap approximation, where inducibility of each induced plant was estimated as the difference between the value of the resin canal trait for a given plant and that of the other four plants of the same family and P treatment in the control treatment (Moreira et al. 2013). This resulted in four estimates of inducibility for each induced plant, which were considered as repeated measures on the same subject. For the statistical analysis of this inducibility trait a repeated measures mixed model, with family, family × P treatment and family × constitutive-block as random factors, was used (Moreira et al. 2013).

In the case of the analyses of growth traits (height and biomass), we used a similar repeated measures approach, analysing the effect of the three main factors (P, F and P × F) on the loss of growth caused by the induction treatment (i.e. on the differences in height and biomass between MJ-induced and control plants). Effects of P availability and genetic variation on growth (i.e. height and biomass) in a larger collection of pine families (N = 34) were reported elsewhere (Moreira et al. 2012b, Sampedro et al. 2011). Here we report the results of the same analyses for the specific material included in the present study, i.e. the 15 *P. pinaster* families.

In order to evaluate the trade-offs between constitutive and induced defences and growth potential, we studied the genetic correlations (family correlations) between pairs of traits. Family correlation between traits is a measure of the degree to which two traits are covariant as a result of pleiotropy or linkage disequilibrium (sensu Agrawal et al. 2010), and negative family correlations evidence a genetic based trade-off between alternative functions that could be relevant for evolutionary dynamics. Family correlations between pine growth and anatomical defences were estimated separately for

the constitutive (control) and MJ-treated (inducibility) treatments across soil P treatments. These correlations were estimated using the best linear unbiased prediction (BLUP) values for each family in each P treatment, as obtained with the mixed models described above for the analyses of the constitutive resin canals and their inducibility.

In order to estimate the relative impact of chemical and anatomical defences on growth reduction, we performed multiple regression analyses for those treatments and variables showing significant genetic variation between the 15 families and significant negative genetic correlations with growth in this study. We also included in the analysis the chemical defensive traits reported by Sampedro et al. (2011) for the families in this study fulfilling the same requirements. Thus, we included in the model the concentration of non-volatile resin in the stems and total polyphenolics in the needles (Sampedro et al. 2011), density of resin canals in the xylem and total density of resin canals as predictors of pine growth.

RESULTS

Effect of exogenous application of MJ on growth and resin canal traits

Exogenous application of MJ significantly reduced plant height and biomass, which were 19% and 18% lower in MJ-treated plants than in control plants (Table SM1, Fig. SM1). Exogenous application of MJ significantly increased the density of resin canals in the cortex (18%), xylem (27%) and the total section (25%), and increased the relative conductive area of canals in the cortex (82%), xylem (66%) and the total section (70%) compared to control plants (Table SM2, Fig. SM2).

Genetic and environmental effects on pine growth in constitutive and MJ-induced conditions

Nutrient limitation strongly affected pine growth in constitutive conditions (Table SM3a, Fig. SM3), but did not alter the reduction in growth caused by the MJ induction treatment (Table SM3b, Fig. SM4). Specifically, in constitutive conditions, total height and biomass of plants grown under P-limited treatment were 39% and 58% lower, respectively, than those under the complete fertilizer (Fig. SM3). Pine families differed significantly in height in constitutive conditions and also differed in the reduction in growth caused by MJ (Table SM3). Variation among pine families in biomass was also significant, but only in constitutive conditions (Table SM3). We did not detect significant family variation in plasticity of growth (height and biomass) in response to soil P availability in the families included in this study (not significant F × P interaction; Table SM3).

Genetic and environmental effects on constitutive resin canals

Availability of P significantly influenced constitutive resin canal traits (Table 1a, Fig. 1a). Density of cortex resin canals and total number of constitutive resin canals was 2.1-fold, and 1.4-fold greater, respectively, in plants grown under the P-limited treatment than in control plants (Fig. 1a, 1e). Constitutive resin canal traits did not significantly differ between families (Table 1a). However, families differed in the phenotypic plasticity of the constitutive density of xylem resin canals in response to P availability as evidenced by a significant F × P interaction (Table 1a; genetic variation in phenotypic plasticity).

Genetic and environmental effects on the inducibility of resin canals

Although the inducibility of resin canals appeared to be consistently lower under complete nutrition than under P-limited conditions in both the xylem and the cortex

(Fig. 2), the differences were not statistically significant in either case (Table 1b). Inducibility of cortex and total relative conductive area and inducibility of xylem resin canal density significantly differed between families (Table 1b). Moreover, significant genetic variation in response to P availability was observed, in terms of the phenotypic plasticity of the induced density and relative conductive area of xylem and total resin canals, and also in the relative conductive area of cortex resin canals (significant F × P interactions; Table 1b).

Genetic correlations between resin canal and plant growth traits

We found a negative family correlation between the density of constitutive resin canals and plant growth, significant for canals in the xylem and for total resin canals, but only under P-limiting conditions (Table 2); this suggests that such growth-defence trade-offs are context-dependent. Family correlations between plant growth and other constitutive resin canal traits were mostly negative, but not significant (Table 2). On the other hand, no significant relationship at the family level between induced resin canal traits and the loss of plant growth associated with the MJ-induction was observed, in either P-limited or complete fertilization treatments (Table 3).

As both anatomical defensive traits (Table 2) and chemical defences (Sampedro et al. 2011) showed significant trade-offs with growth rates, we used a multiple regression approach for identifying which defensive trait had the greatest effect on growth (see Table SM4). Despite both resin canals and the concentration of non-volatile resin being significantly related to pine growth at the family level when analyzed separately, resin ducts were the only trait that were significant when included in a multiple regression model, explaining the largest part of the variation in family growth (Table SM4).

DISCUSSION

Results showed that nutrient availability strongly influenced the early resource allocation to constitutive anatomical defences in *P. pinaster*, whereas it had no effect on their inducibility. In particular, the density of constitutive resin canals in the cortex and in the whole stem section was higher under P-limiting conditions than under complete fertilization. Nutrient availability also influenced the extent of vegetative costs associated with plant defensive investment. Our results showed genetic based trade-offs between the formation of constitutive resin canals and plant growth, which only emerged under P-limited conditions. Finally, we found genetic variation in the inducibility of some resin canal traits, and in the response of most inducible resin canal traits to P availability (i.e. significant Family × P availability interaction).

Effects of soil P availability on constitutive resin canals

Plasticity of the constitutive resin canals observed in the cortex in response to nutrient availability was also recorded in several other field and greenhouse studies of conifers (e.g. vanAkker et al. 2004, Moreira et al. 2008). In an extensive field study performed in NW Spain, unfertilized 2-year-old seedlings of *P. pinaster* showed up to 30% more resin canal density in the cortex than the fertilized seedlings, whereas soil fertilisation had no effect on the resin canal system in the xylem (Moreira et al. 2008). In another field experiment performed in British Columbia (Canada), vanAkker et al. (2004) reported that fertilisation significantly reduced constitutive resin canal density of 12-year-old *Picea glauca* x *P. engelmannii* hybrids. Results also agree with previous work reporting increased accumulation of chemical defences under impoverished nutrient conditions. Particularly, in a greenhouse experiment using the same plant material as in

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408the current study, Sampedro et al. (2011) observed that pine seedlings growing under P-
409limited conditions had higher concentrations of constitutive stem resin, needle total
410phenolics and needle condensed tannins than plants growing under complete
411fertilisation. Overall, these results support the predictions of ecological theories (e.g.
412GDBH, RAH) about the effects of limited resource availability on the allocation to
413constitutive defences of plants (Stamp 2003). These theories predict that due to
414physiological trade-offs between plant growth and secondary metabolism investment,
415and due to the high value of tissues when growth is slow, plants growing under limited
416resource conditions should be highly protected by constitutive defences. Our results
417regarding greater constitutive resin canals under nutrient deprivation may provide a
418mechanistic explanation for understanding the results of several field studies reporting
419negative effects of stand fertilization on conifer tree resistance to phloem-feeders (e.g.
420vanAkker et al. 2004, Zas et al. 2005, Zas et al. 2006).
421
422Effects of soil P availability on the inducibility of resin canal traits
423Induction of resin canals after MJ application has been previously reported in several
424conifer species, as MJ application is known to be a useful chemical treatment
425mimicking herbivore-induced responses. In particular, extensive formation of traumatic
426resin ducts in the xylem in response to MJ has been reported for *Picea abies*
427(Franceschi et al. 2002, Martin et al. 2002, Krokene et al. 2003, Krokene et al. 2008)
428and *Picea sitchensis* (Miller et al. 2005), but no previous studies have analysed the
429effect of MJ on the canal system of pine species. Nonetheless, pine trees are known to
430respond to herbivore feeding by producing traumatic resin ducts in the xylem (Moreira
431et al. 2008). On the other hand, the increase in resin canal density in the cortex after MJ
432application was probably due to a decrease in the cortex area rather than an increase in

433the number of resin canals. Resin canals in the cortex are known to be altered by biotic
434challenges in other conifer species (Kuroda, 1998), but again no previous information is
435available for pine species.

436 In contrast with constitutive resin canals, our results show that inducibility of
437resin canal differentiation was not dependent on nutrient availability. Accordingly,
438using the same experimental plants, Sampedro et al. (2011) found that inducibility of
439non-volatile resin in juvenile pine trees was similar in both P treatments. In the same
440study, however, Sampedro et al. (2011) found that inducibility of total polyphenolics
441was significantly greater under P-limited conditions. Contrastingly, other studies with
442annual plants have reported the opposite pattern, with increased inducibility of
443chemical defences under improved growing conditions. For example, Dietrich et al.
444(2004) reported that *Arabidopsis thaliana* showed significant induced production of
445total soluble proteins only when cultivated under high nitrogen conditions. We think
446that due to their different life-history determinants, different modes of defence
447(quantitative vs. qualitative, respectively), and the different nature of their chemical
448defences (e.g. carbon- vs. nitrogen-based), the influence of nutrient availability on
449induced defences of long-lived pine trees and herbaceous plants are probably not
450comparable.

451

452Effects of soil P availability on the costs associated with constitutive and induced resin
453canals

454Our results show that the costs associated with constitutive resin canal formation were
455contingent upon the availability of P in the soil. Most notably, a genetic based trade-off
456between the density of constitutive resin canals in the xylem (and in the total section)
457and plant growth occurred only under limited availability of P. Fitness costs associated

458with the production of constitutive chemical defences under resource-limited
459environments have been commonly observed in a diverse array of long-lived tree
460species (e.g. Lindroth et al. 2001, Donaldson et al. 2006, Osier and Lindroth 2006,
461Donaldson and Lindroth 2007, Sampedro et al. 2011), but to the best of our knowledge
462this is the first study showing early fitness costs associated with constitutive anatomical
463defences in a tree species. Specifically for *P. pinaster*, in a previous study with the
464same experimental plants, we found that growth rates were lower in pine families
465showing the highest concentrations of constitutive non-volatile terpenes, again only
466under P limited conditions (Sampedro et al. 2011). Using a multiple regression
467approach we found that production of resin canals provoked higher reductions in
468growth than chemical defences (see Table SM4). This result was expected, as the
469production of resin canals in conifer trees is a more energy-costly process (based on
470cell division and differentiation) than the production of chemical defences (based on
471local changes in cell metabolism) (Bonello et al. 2006).

472 No vegetative costs were associated with the inducibility of resin canals in
473either P-limited or complete fertilization. This finding does not agree with our previous
474work reporting a negative family correlation between induced chemical defences and
475plant growth under P-limited conditions (Sampedro et al. 2011). One possible
476explanation for this is that the production of resin canals in conifer trees is a slower
477process than the production of chemical defences (Bonello et al. 2006); if more than
478two weeks had elapsed between the induction treatment and the assessment of growth,
479vegetative costs may well have been detected. Other previous studies using conifer
480(Villari et al. 2014) and herbaceous (e.g. van Dam and Baldwin 1998) plants reported
481either no growth costs associated with induced defences, or that fitness costs arose only
482under rich-resource conditions (e.g. Van Dam and Baldwin 2001, Cipollini 2010).

Further research is needed to derive general patterns about fitness costs associated with the inducibility of defences and to determine (i) which defensive compounds are more or less canalized, (ii) the magnitude of allocation costs they generate, and (iii) under what environmental conditions fitness costs are magnified.

Genetic variation in constitutive and induced anatomical defences and in their response to P availability

We found genetic variation in the inducibility of changes in the relative conductive area of cortex and total resin canals, and also in the inducibility of increases in xylem resin canal density, but not in constitutive resin canals. These results agree with our previous observations in young pine trees (Sampedro et al. 2011, Moreira et al. 2013), where we found genetic variation in the inducibility of diterpenes in the stem and total phenolics in the needles (Sampedro et al. 2011, Moreira et al. 2013). Similarly, Agrawal et al. (2002) also found genetic variation in the induction of chemical and mechanical defences in wild radish. Genetic variation in the inducibility of plant defences indicate that defensive strategies have the potential to evolve further in response to continued herbivore pressure.

We also found genetic variation in the response of most inducible resin canal traits (density and relative conductive area of xylem and total resin canals and relative conductive area of cortex resin canals) to P availability (i.e. significant Family × P availability interaction). Although genetic variation in the response of constitutive defences to environmental conditions has been commonly observed in tree species (e.g. Donaldson et al. 2006, Osier and Lindroth 2006, Donaldson and Lindroth 2007), little is known in the case of inducible defences. Further studies should be undertaken to fill this gap in our knowledge, as genetic variation in the response of inducible defences

allows the continued evolution of phenotypic plasticity, i.e. the ability of an individual or genotype to express different phenotypes across varying environmental conditions.

Implications for forest management

Recent studies have provided evidences that resin canals are reliable proxies of pine tree resistance against pests and pathogens. For example, Kane and Kolb (2010) observed that ponderosa pine trees that survived from drought-associated bark beetle attacks in forests of northern Arizona had larger and denser resin canal ducts in the xylem than those that died. Similarly, Ferrenberg et al. (2014) found that pine trees resistant to bark beetles had significantly more resin ducts in the xylem than susceptible trees. Resin duct characteristics could, thus, offer a metric for assessing tree resistance and predicting the spatial/temporal spread of pests such as bark beetles during epidemic events (e.g. Gaylord et al. 2013). From an applied point of view, elicitation of inducible resin canals by MJ application to seedlings in the nursery could be an attractive measure to protect forest plantations from early biotic challenges. In this sense, Zas et al. (2014) recently found that exogenous MJ application to young pine trees in the nursery markedly increased the concentration of non-volatile resin in the stems and drastically reduced the subsequent attack of a phloem-feeder weevil (*Hylobius abietis*). As resin canals are known to be major proxies of pine tree resistance to pests and pathogens, understanding defensive allocation to resin canals, their genetic variation and environmental modulation could be considered in managing forest tree resistance and ultimately in forest management practices.

Conclusions

As previously reported for chemical defences (Sampedro et al. 2011), this work reveals that nutrient availability (particularly that of P, a major growth-limiting nutrient for the studied population) is a major driver in the differentiation of anatomical defences in young *P. pinaster*. Availability of P in the soil had significant effects on resource allocation to constitutive resin canals, the induction of canal formation and the emergence of related vegetative costs. Because insects are a major cause of early mortality in *P. pinaster*, our findings are of great relevance for understanding the strategies used by this light-demanding pioneer tree against herbivory during its early life stages (Zas et al. 2005, Zas et al. 2006).

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Table 1. Effects of phosphorus availability, pine family and their interaction on constitutive resin canal traits and on their inducibility. Summary of mixed models analysing the effect of phosphorus-limitation on several traits describing the resin canal system of 15 *Pinus pinaster* open-pollinated families in a) constitutive mode and b) herbivore-induced responses associated to jasmonate signalling (inducibility). Phosphorus availability (P) is a fixed effect, and the *F*-values and corresponding *df* are shown. The family effect (Fam) and P × Fam interaction are random effects, and the corresponding likelihood ratio significance tests (χ^2) are shown. Significant *P* values ($P \leq 0.05$) are typed in bold.

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Table 2. **Genetic based trade-offs between early growth and constitutive resin canal traits.** Pearson's *r* coefficients (and significance levels) from family correlations between several traits of the constitutive resin canal system and plant growth in young *Pinus pinaster* grown under P-limiting and complete fertilization conditions. Negative significant family correlation evidence a heritable inverse genetic co-variation between growth and defence. Observed trade-offs were context dependent, as they were not evident under complete fertilization. Correlations were estimated using the best linear unbiased prediction (BLUP) values for each family and P treatment. Significant coefficients ($P < 0.05$) are in bold. N = 15 open-pollinated families.

	Phosphorus availability (P)		Family (Fam)		Fam × P	
	$F_{1,3}$	*P*	χ^2	*P*	χ^2	*P*
a) Constitutive						
Cortex resin canal density	25.11	**0.015**	-	-	-	-
Cortex resin canal relative conductive area	5.26	0.105	-	-	-	-
Xylem resin canal density	0.50	0.532	-	-	3.9	**0.024**
Xylem resin canal relative conductive area	6.55	0.083	0.7	0.201	0.6	0.219
Total resin canal density	23.88	**0.016**	-	-	1.0	0.159
Total resin canal relative conductive area	0.00	0.985	1.9	0.084	0.1	0.376
b) Inducibility						
Cortex resin canal density	4.46	0.125	-	-	0.4	0.263
Cortex resin canal relative conductive area	1.68	0.285	13.9	**<0.001**	13.0	**<0.001**
Xylem resin canal density	1.87	0.265	3.8	**0.026**	16.8	**<0.001**
Xylem resin canal relative conductive area	1.33	0.333	1.0	0.159	18.3	**<0.001**
Total resin canal density	6.87	0.079	2.2	0.069	8.0	**0.002**
Total resin canal relative conductive area	3.54	0.156	5.8	**0.008**	20.1	**<0.001**

	P-limited		Complete fertilization	
	Height	Biomass	Height	Biomass
Cortex resin canal density	–[a]	–	–	–
Cortex resin canal relative conductive area	–	–	–	–
Xylem resin canal density	**-0.631** **(0.021)**	0.409 (0.164)	0.303 (0.272)	0.017 (0.951)
Xylem resin canal relative conductive area	-0.207 (0.459)	-0.007 (0.980)	0.007 (0.981)	-0.217 (0.438)
Total resin canal density	**-0.769** **(0.001)**	0.289 (0.296)	0.282 (0.309)	0.129 (0.647)
Total resin canal relative conductive area	-0.170 (0.545)	-0.326 (0.236)	-0.172 (0.540)	-0.320 (0.245)

[a]Because of null estimations of family and P x Family variances the corresponding blups are equal zero.

Table 3. **Family based Pearson's r coefficients (and significance levels) between early growth and inducibility of resin canal traits.** Pearson's *r* coefficients (and significance levels) from family correlations between several traits describing the inducibility of resin canal system and the reduction in plant growth due to the induction treatment in young *Pinus pinaster* grown under P-limiting and complete fertilization conditions. These correlations were estimated using the best linear unbiased prediction (BLUP) values for each family and P treatment. Significant coefficients ($P < 0.05$) are in bold. N = 15 open-pollinated families. Herbivore-induced responses were elicited by exogenous application of methyl jasmonate.

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	P-limited		Complete fertilization	
	Height	Biomass	Height	Biomass
Cortex resin canal density	-0.244 (0.381)	0.065 (0.819)	0.209 (0.454)	-0.165 (0.556)
Cortex resin canal relative conductive area	-0.162 (0.564)	0.409 (0.146)	-0.300 (0.277)	0.438 (0.103)
Xylem resin canal density	0.388 (0.153)	-0.281 (0.311)	0.459 (0.085)	0.260 (0.348)
Xylem resin canal relative conductive area	0.388 (0.152)	-0.021 (0.942)	-0.066 (0.816)	0.410 (0.129)
Total resin canal density	0.307 (0.265)	-0.332 (0.227)	0.358 (0.190)	-0.000 (0.999)
Total resin canal relative conductive area	0.239 (0.392)	0.299 (0.279)	-0.306 (0.267)	0.264 (0.341)

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742**FIGURE LEGENDS**

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744**Figure 1. Effect of phosphorus availability on constitutive resin canal.** Resin canal 745density and relative conductive area of constitutive resin canals in the cortex (a, b), 746xylem (c, d) and the whole stem section (e, f) of *Pinus pinaster* juveniles belonging to 74715 open-pollinated families growing in a nutrient-rich (complete fertilization) and in P-748limited media. Bars are lsmeans ± s.e. (N = 60). Results of the mixed model are 749presented in Table 1a. Different letters indicate significant differences (at $P < 0.05$) 750between fertilization treatments.

Figure 2. Effect of phosphorus availability on the inducibility of resin canal traits. Inducibility of resin canal density and relative conductive area in response to herbivore-damage signalling in the cortex (a, b), xylem (c, d) and the whole stem section (e, f) of *Pinus pinaster* juveniles belonging to 15 open-pollinated families growing in a nutrient-rich (complete fertilization) and in P-limited media. Bars are lsmeans ± s.e. (N = 60). Results of the mixed model are presented in Table 1b. Herbivore-induced responses were elicited by exogenous application of methyl jasmonate.

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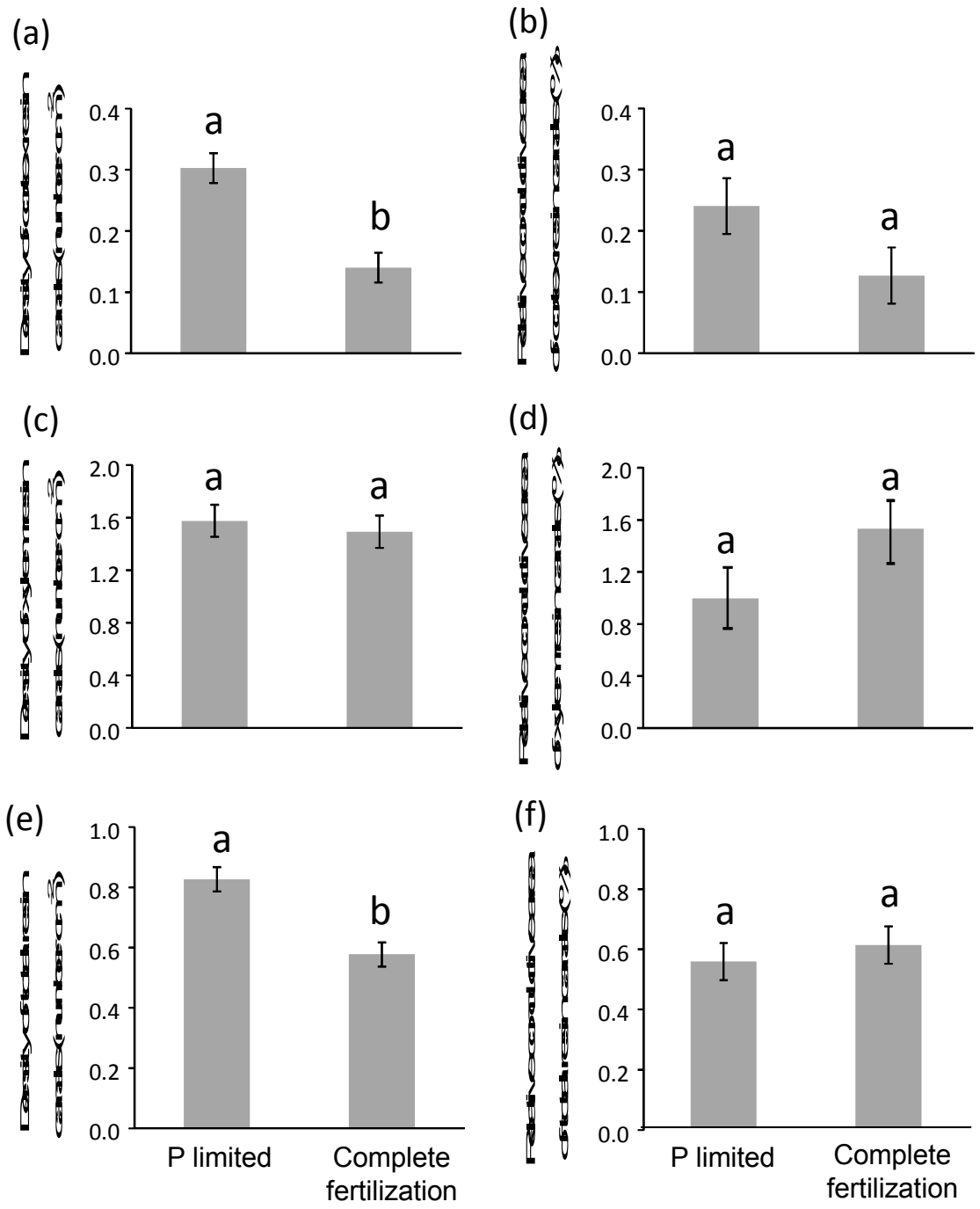

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771**Figure 1.** Moreira et al

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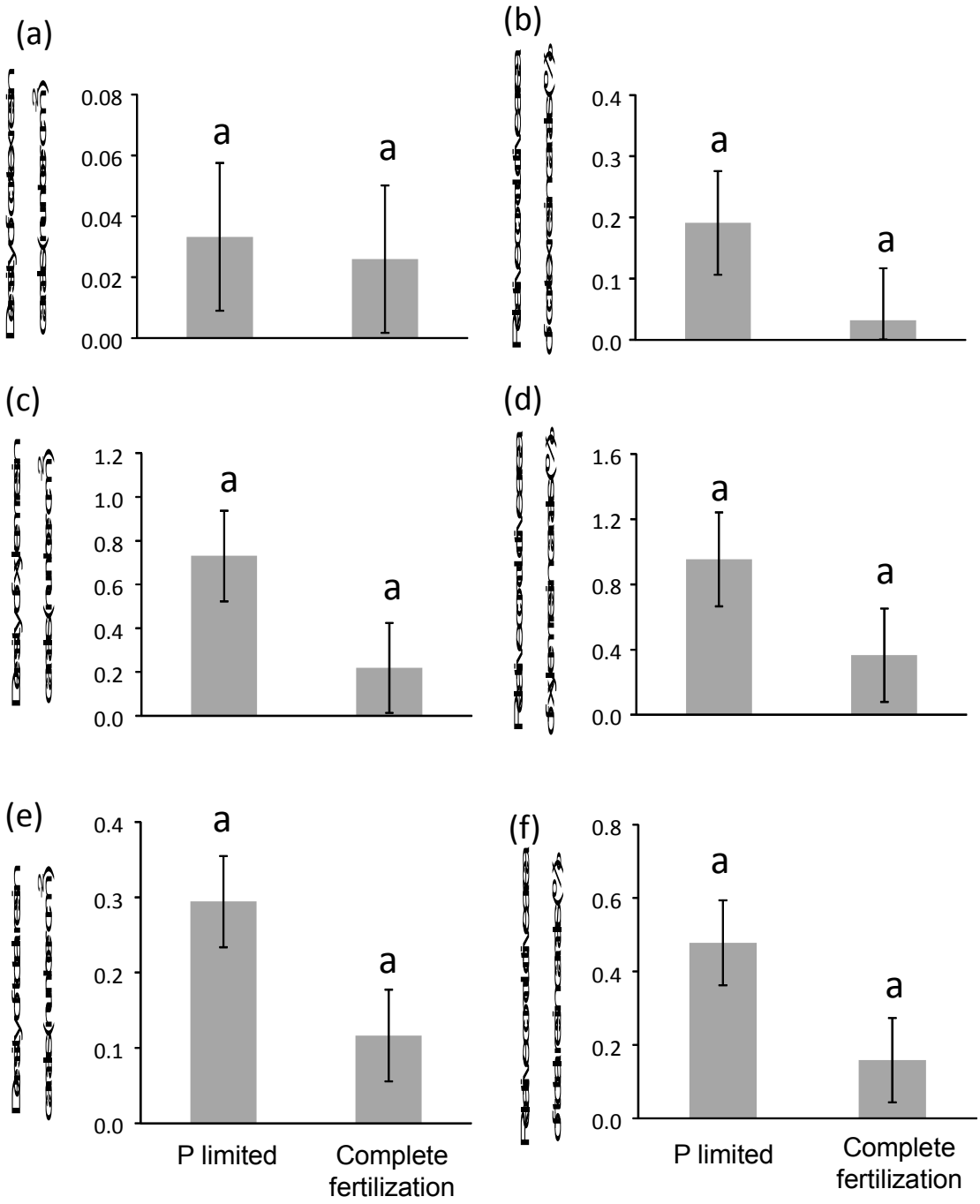

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778**Figure 2.** Moreira et al

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